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EFFECTS OF SALINITY ON FERTILIZATION SUCCESS IN TWO POPULATIONS OF *FUNDULUS HETEROCLITUS*

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ABSTRACT

A population of killifish (*Fundulus heteroclitus*) from northern NJ (Newark Bay and Piles Creek, salinity ~20‰) produces fertilizable ova only if stripped into reduced salinity water, while populations from estuarine areas of Long Island (Montauk and Southampton, salinity 20–25‰) produce viable eggs over a range of salinities (10–30‰). It was impossible to fertilize Piles Creek eggs in 30‰ even if sperm were already present in the water, but if eggs were transferred from 30‰ to 15‰ within one minute, successful fertilization was obtained. In full strength sea water (30‰) they became artificially activated and produced 90–100% noncleaving eggs, whereas Long Island eggs did not become artificially activated even after two hours in 30‰ salinity. The salinity to which sperm were initially exposed did not appear to affect their ability to fertilize ova from either population.

When Piles Creek eggs were stripped into 30‰ salinity in the absence or presence of sperm, SEM observations showed that the micropyles became blocked with extrusions seeming to originate from within the micropylar canal, and in some cases with cortical granules. This blockage very closely resembled the micropylar blockage seen in eggs after fertilization at 15‰ salinity. Ova stripped into 15‰ salinity without sperm generally had no micropylar blockage. This suggests that in the Piles Creek population, contact with full strength sea water rapidly initiates artificial activation, resulting in the blockage of the micropyle.

INTRODUCTION

Many investigators have addressed the issues of the fertilization process and artificial activation of the teleost ovum. Kagan (1935) noted that the fertilizable life of a *Fundulus heteroclitus* egg was 15–20 minutes. Beyond this time the percentage of successfully fertilized eggs became very small, because eggs became artificially activated, developing a perivitelline space after about 20 minutes in sea water. Kagan attributed the activation of the eggs to the sea water and concluded that the eggs which developed the perivitelline space (became activated) before insemination could not become fertilized. An artificially activated egg may form a one-cell stage, but it does not divide.

In teleost fertilization the sperm enters the ovum through a micropyle, which in *Fundulus* consists of a single opening in the chorion (Brummett and Dumont, 1981a) at the bottom of a slight depression. The micropyle apparatus consists of a relatively smooth sided funnel-shaped entrance, at the bottom of which is a 4–5 μ diameter pore, surrounded by slightly elevated lips. The micropylar canal diminishes in diameter (to 2–3 μ) as it approaches the plasma membrane of the ovum

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Abbreviations: NCE–noncleaving eggs, PC–Piles Creek, SH–Southampton.

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(Kuchnow and Scott, 1977). Although the outer diameter of the micropylar funnel permits the entry of several sperm, the diameter of the inner opening allows only one sperm access to the egg (Brummett and Dumont, 1979).

Brummett and Dumont (1981a) in their ultrastructural studies on fertilization report that upon activation a "fertilization plug" is formed, sealing the inner opening of the micropyle and effectively preventing polyspermy. They speculated that artificially activated eggs might undergo the same micropylar blockage as eggs activated by fertilization. They found that cortical vesicle breakdown occurring upon activation could be somewhat explosive, liberating membrane fragments into the perivitelline fluid. Fibrous and particulate material as well as dense spheres were shown to be liberated from the wave of cortical vesicle breakdown. The breakdown of the cortical vesicles is a major structural change which might be the origin of the block to fertilization that is seen after artificial activation has occurred.

Fundulus heteroclitus is a common estuarine species, living in water ranging from fresh water to about 30‰ salinity. While studying pollution tolerance in embryos from Newark Bay and from Piles Creek in Linden, NJ, and from Montauk and Southampton, NY, it was discovered that when eggs of the Newark Bay or Piles Creek females (from ~20‰ salinity water) were stripped into 30‰ salinity water and mixed with sperm, the batches obtained would consist primarily of non-cleaving eggs (Weis *et al.*, 1981). Successful development would occur, however, if eggs were stripped into 15‰ salinity. Montauk or Southampton eggs (from sites of 20–25‰ salinity) could become fertilized and develop successfully at any salinity tested (10–30‰).

The present study is an attempt to analyze the unique response to sea water seen in the northern NJ ova in terms of timing, specific salinity, and morphological changes in the eggs.

Materials and Methods

Mature, gravid *Fundulus heteroclitus,* collected at Piles Creek (PC) in Linden, NJ, or Southampton (SH), NY from May through July, were transported to the laboratory where they were separated by sex and maintained in aerated, filtered, diluted sea water (17–21‰ salinity) for several days. Ripe ova were obtained by stripping gravid females into finger bowls with sea water of a specific salinity. The sea water was prepared by dissolving artificial sea salts ("Rila Mix") in aged tap water and aerating the mixture overnight. All experiments were done at room temperature (~24°C). The ova were inseminated with milt stripped from males. Enough males were used in each experiment to assure an adequate supply of sperm. The experiments were designed so that the response of ova (from both populations) to different salinities could be determined.

(1) In the first experiment 602 ova from a single PC female were stripped and divided into two groups. Of these, 243 ova were inseminated in 30‰ salinity water with sperm from 3 PC males, and were maintained at this salinity for 5, 10, 15, 20, or 25 minutes, or until a one-cell stage had formed (approximately 60 minutes). They were then rinsed in 15‰ water and placed into 15‰ salinity water to develop. Development was considered to proceed normally if the blastodisc cleaved. In each group, the non-cleaving eggs (NCEs) were counted and the percentage of NCEs calculated. The reverse of this experiment was carried out simultaneously with the second group (359) of these eggs; *i.e.* the eggs were inseminated in and maintained at 15‰ salinity for corresponding time intervals before being transferred to 30‰ salinity. Similarly, 608 ova from a single SH female were divided into two groups,

and treated as above, except that they were inseminated with sperm from 3 SH males.

This experiment was repeated with 100 eggs from a PC female transferred from a 30‰ sperm suspension to 15‰, and another 100 eggs transferred from a 15‰ sperm suspension to 30‰ after 1, 2, 3, 4, and 5 minutes.

(2) To determine if 30‰ salinity activated the PC ova, eggs were stripped and were allowed to sit in 30‰ for up to three hours without sperm. Some were removed after 5 and 10 minutes, rinsed, and inseminated at 15‰ with milt from PC males. Other ova from the same females were maintained for up to three hours at 15‰ without sperm. Some of these were also removed at 5 and 10 minutes, rinsed, and inseminated with sperm from PC males in 30‰ salinity. A total of 201 ova from three females were used in these experiments.

(3) To determine the approximate salinity at which the PC ova would be fertilizable, ova were inseminated at 15‰, 20‰, 25‰, and 30‰ salinity. One such run was carried out using 77 ova stripped from a single PC female, inseminated with sperm from one PC male.

(4) To determine if exposure to either 30‰ or 15‰ salinity water would affect the ability of sperm to fertilize the ova, milt was stripped from PC males into a small volume of water of 15‰ or 30‰ salinity. Ova were stripped from PC females into separate finger bowls containing 30‰ and 15‰ salinity water respectively. The sperm suspension was then immediately added to the finger bowl containing the ova. (This would have changed the salinity slightly.) Sperm from SH males were also used in attempting to fertilize PC ova at 30‰ and 15‰ salinity. Ova from the SH population were used in the control runs. In a final experiment, milt was stripped from PC males into 30‰ salinity, and PC ova were stripped directly into the sperm suspension.

Specimens to be examined with the SEM were preserved in 0.07 *M* phosphate-buffered 4% glutaraldehyde solution for 2 and 3 days, after which they were dehydrated through a series of alcohols (30–100% EtOH) and acetone at 10-minute intervals. After dehydration, the specimens were critical point dried from CO_2 using a SAMDRI 960 (Tousimis) Critical Point Drier, and mounted on stubs with silver paint or double-stick tape and gold coating. The specimens were both inseminated and uninseminated eggs from PC females stripped into both 15‰ and 30‰ salinity. The durations of exposure of these ova to a given condition were 30 seconds, 2 minutes, and 3 minutes.

Forty ova were observed with a Scanning Electron Microscope (ISI IIIA) operated at 30 kV.

Results

Population response to salinity

(1) The first experiment revealed that the effects of salinity on *Fundulus* ova occur within the first five minutes of contact with the medium. Table I shows that only the ova from the PC population are incapable of being successfully fertilized in full strength sea water. In 30‰ salinity water PC ova developed only up to the one-cell stage. Occasionally, however, these eggs did not form a perivitelline space or a one-cell stage. Regardless of whether an egg formed a perivitelline space, if it did not cleave it was included in the calculation of percentage NCEs. The SH ova, inseminated with SH sperm, were generally successfully fertilized (Table I) regardless of the salinity of the medium in these experiments.

TABLE 1

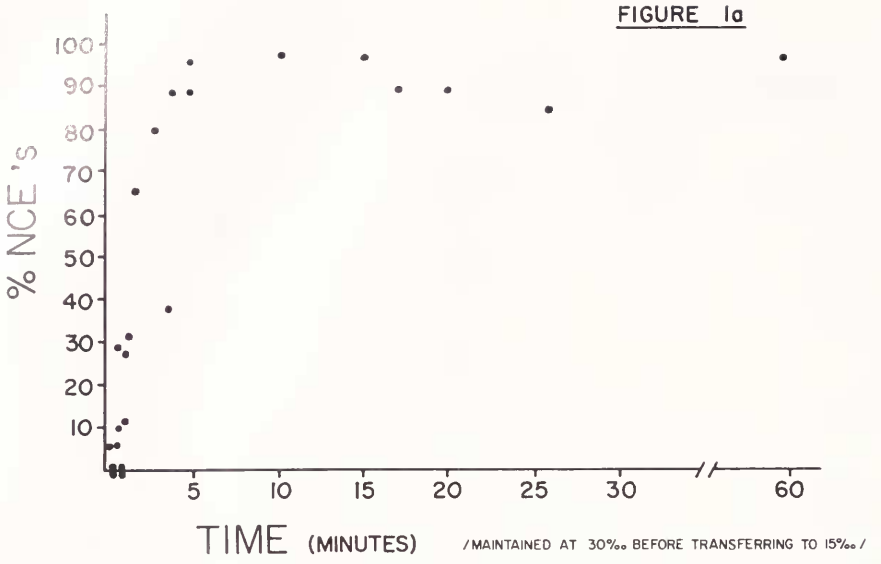
Results of experiments in which F. heteroclitus *ova are inseminated at one salinity and after a given interval of time are transferred to a second salinity*

Parent fish (male × female)	Fertilization salinity (FS) (‰)	Time spent in FS (minutes)	Final salinity (‰)	# of Ova	%NCE's
PC × PC	15	5	30	55	2
PC × PC	15	10	30	51	0
PC × PC	15	15	30	31	0
PC × PC	15	20	30	26	0
PC × PC	15	25	30	28	0
PC × PC	15	indefinite (~60)	15	52	0
PC × PC	30	5	15	103	95
PC × PC	30	10	15	77	97
PC × PC	30	15	15	40	97
PC × PC	30	20	15	34	95
PC × PC	30	25	15	50	86
PC × PC	30	indefinite (~60)	30	55	95
SH × SH	15	5	30	42	0
SH × SH	15	10	30	51	2
SH × SH	15	15	30	37	5
SH × SH	15	20	30	92	1
SH × SH	15	25	30	74	0
SH × SH	15	indefinite (~60)	15	47	4
SH × SH	30	5	15	73	10
SH × SH	30	10	15	57	2
SH × SH	30	15	15	41	0
SH × SH	30	20	15	40	0
SH × SH	30	25	15	61	2
SH × SH	30	indefinite (~60)	30	53	6

When determining if ova would cleave when removed from a 30‰ sperm suspension after a shorter period of time, it was observed that the PC ova were successfully fertilized if transferred from 30‰ to 15‰ within the first minute of exposure to 30‰ (Fig. 1a). It was also found that ova transferred from 15‰ to 30‰ within one minute produced a very high percent of NCEs (Fig. 1b). Figures 1a and 1b also include data from the longer exposures done initially.

(2) The second series, designed to discover whether the PC ova became activated and thus unfertilizable when exposed to 30‰ for 5 or 10 minutes before insemination at 15‰, resulted in 81% and 100% NCEs respectively. When fertilization was attempted at 30‰ after exposure to 15‰ for 5 and 10 minutes, 100% and 90% NCEs were obtained. However, if inseminated in the 15‰ salinity after comparable periods, fertilization was successful with almost no NCEs (3%). Such data are consistent with previous results.

(3) The investigations of what critical salinity prevented fertilization revealed that successful fertilization could occur at 15‰, 20‰, and 25‰ salinity (Fig. 2) although at 25‰ some reduced success was evident. These data are taken from eggs of one female, however, and there is probably some variability within the population.



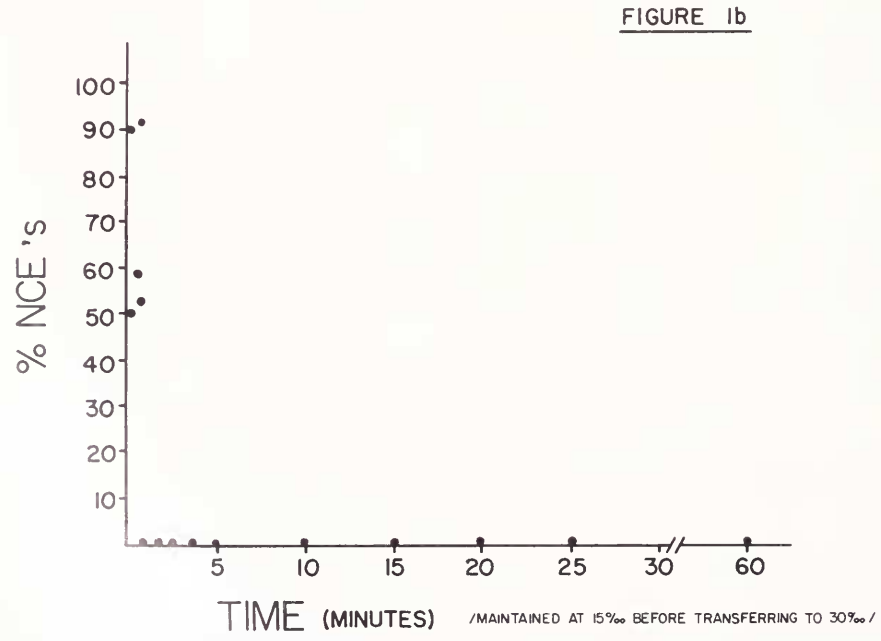


FIGURE 1. a) Percentage of noncleaving eggs (%NCE's) obtained if a batch is allowed to remain in 30‰ salinity with sperm for a given time interval before being transferred to 15‰ salinity. Each point represents a single batch of eggs (20–40 ova) observed. b) %NCE's obtained if a batch is allowed to remain in 15‰ salinity with sperm for a given time interval before being transferred to 30‰ salinity.

(4) In the experiments determining if exposure of sperm to different salinities would affect their ability to fertilize PC ova, it was found that the exposure of sperm of either population to various salinities did not affect their ability to successfully fertilize ova. An average of 73% NCEs was obtained from 4 replicate experiments

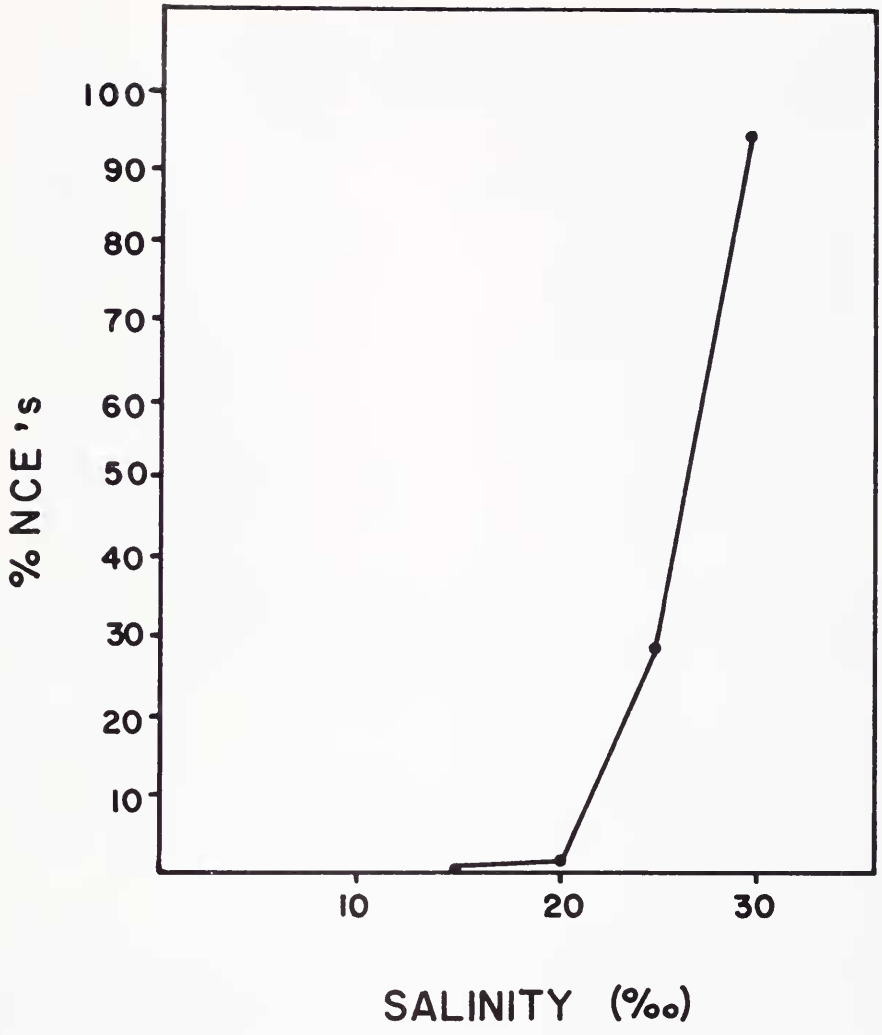


FIGURE 2. %NCE's as a function of the salinity.

in which PC sperm were exposed to 15‰ for one minute and then used to inseminate ova at 30‰ salinity. When this same procedure was performed with a reversal of the salinities (sperm in 30‰, eggs in 15‰) most of the eggs cleaved successfully, with the NCEs averaging 7% in six replicates. When sperm from SH males was used to inseminate PC ova at either salinity, the results were comparable. When eggs were at 30‰ and sperm at 15‰ salinity the NCEs averaged 65% in four replicates, and when eggs were at 15‰ and sperm at 30‰ salinity the NCEs averaged 5% in four replicates. SH eggs in 15‰ or 30‰ salinity inseminated with sperm from either PC or SH males all averaged less than 10% NCEs. In the final experiment, in which PC ova were stripped directly into a sperm suspension in 30‰ salinity, 88% NCEs were obtained. Ova from the same female were sucessfully fertilized (7% NCEs) using the above technique in 15‰ salinity sea water.

SEM observations of ova

SEM views of the ova at low magnification (Fig. 3) show the micropyle as a conical depression in the surface. Detailed observations on micropylar regions of 40 ova which were exposed to different salinities for varying intervals of time showed the following results: Exposure of ova to 15‰ salinity without sperm for up to three minutes resulted in 60% of the micropyles remaining open. Figure 4 depicts one such open micropylar canal. There is no evidence of extrusions of any kind that would lead to blockade of the canal and, thus, exclusion of sperm.

When PC ova were inseminated in 15‰ salinity, 92% of the micropyles were blocked by what seemed to be extrusions from within the micropylar canal. Figure 5 illustrates a type of obstruction that is particulate in nature. Figure 6 illustrates what seems to be the release of cortical granules (2.75 μ in diameter) through the opening of the micropyle. All SEM pictures in this set are of ova removed after 30 seconds of exposure to a 15‰ salinity solution containing sperm.

In 90% of the ova observed under conditions of 30‰ and salinity and the absence of sperm, particulate, fibrillar, or spherical blocks formed around the micropyle within 30 seconds of exposure (Fig. 7). Figure 7 also illustrates an interesting ob-

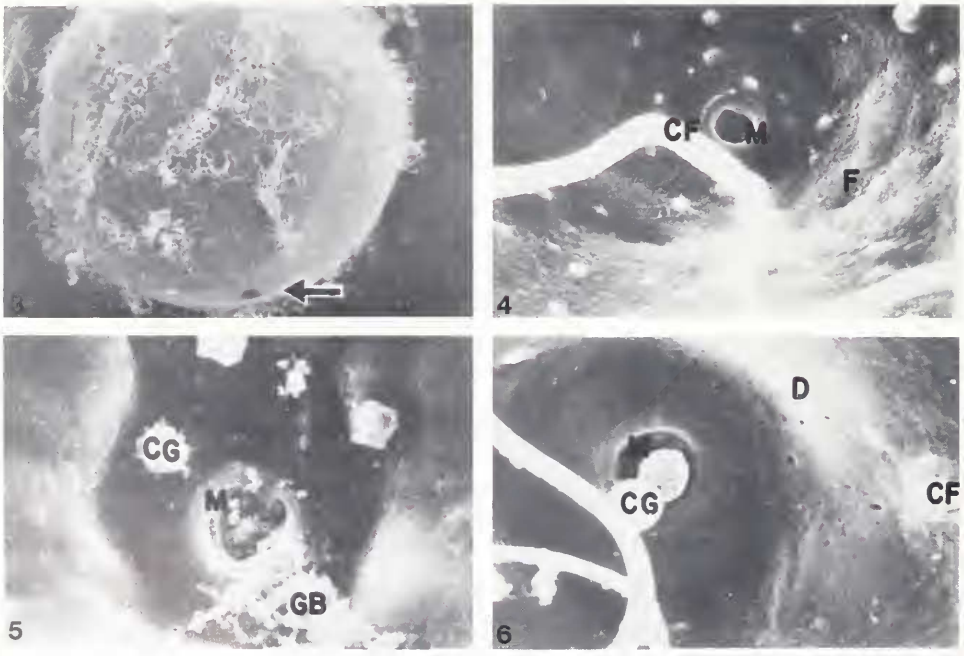


FIGURE 3. Low magnification SEM of an ovum fixed immediately after shedding. Arrow points to the location of the micropyle. Long chorionic fibers are present on the surface of the ovum. (Magnification ×50)

FIGURE 4. A SEM view of the micropyle (M) on the outer surface of the ovum fixed after 30 s in 15‰ salinity without sperm. No chorionic fibers (CF) are present on the inside of the funnel (F) leading to the micropyle. The elevated lip is visible in this picture. (Magnification ×2000)

FIGURE 5. A micropyle (M) obviously blocked by cortical granules (CG) and granular extrusions (GB); ovum inseminated at 15‰ salinity and fixed at 30 s after insemination. (Magnification ×4000)

FIGURE 6. Cortical granules (CG) being extruded from within the micropyle; ovum inseminated in 15‰ salinity and fixed 30 s after insemination. Chorionic fibers (CF) are visible at the edges of the depression (D) leading to the micropyle. (Magnification ×4000)

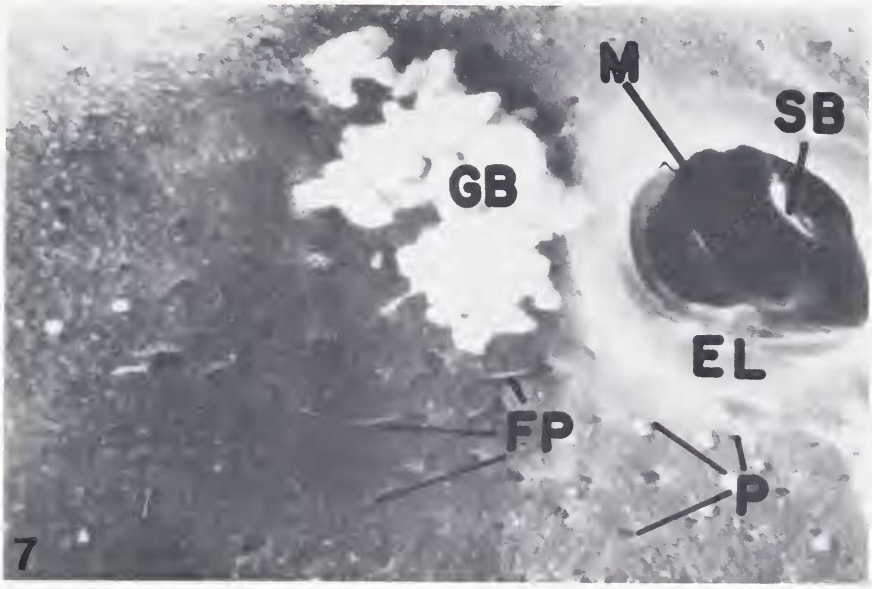


FIGURE 7. An elevated lip (EL) is visible surrounding the micropyle (M). There are pores (P) around the micropyle with fibrillar particles (FP) extending out of them. Ovum was fixed after 2 min exposure to 30‰ salinity with no sperm. Granular extrusions (GB) and a spherical block (SB) are visible. (Magnification ×5000)

servation. Pores are present around the micropyle, and fibrils can be seen extending out of them. Such pores were observed to be present around all micropyles that could be closely examined, but the fibrils were not always present in these pores. Our data reveal no consistent pattern of presence or absence of these fibrils.

Finally, ova subjected to insemination at 30‰ salinity revealed blockage of the micropyles in 100% of the specimens observed. Blockage was very similar to that observed under all the other conditions: the blocks were either spherical, fibrillar, or granular. Figure 8 illustrates blockage at 30‰ salinity in the presence of sperm. There is no way to determine if this egg was artificially activated or successfully fertilized, although, judging from experimental data, most such eggs were artificially activated by the water.

DISCUSSION

The data reveal a striking difference in the ability of ova of the two populations to become fertilized at 30‰ salinity and no difference if fertilization was attempted at 15‰ salinity. The PC population could not produce viable gametes at 30‰, even if eggs were stripped directly into a sperm suspension. Only if they were changed from 30‰ to 15‰ salinity within one minute would successful development occur.

Rao (1974) studied the influence of salinity on fertilization of *Fundulus parvipinnis* inhabiting the bays, lagoons, and fresh water areas along the southern California coast. He found successful fertilization to occur within the salinity range of 5‰ to 33‰, or those salinities that constituted the natural conditions for this species.

F. heteroclitus lives in fresh water, sea water (30‰–34‰) and all intermediate salinities (Feldmeth and Waggoner, 1972). Therefore, one might expect that they should be able to produce viable offspring in a wide range of salinities. The SH

FIGURE 8. An ovum fixed 3 min after insemination in 30‰ salinity. Total blockage of the micropyle (M) has occurred. (Magnification ×3600)

population lives in a less than full strength sea water habitat (20–25‰), and produces viable offspring at varying salinities. The PC population (~20‰) however, could not produce viable offspring at 30‰ salinity.

Holliday (1971) reported that crossfertilization between the races of fish species results in responses characteristic of the female parent. In our observations, cross-fertilization between the two populations similarly resulted in a response to salinity that was characteristic of the origin of the female. The ability of sperm to fertilize ova was not affected by the salinities tested.

Brummett and Dumont (1979) found that activation of the *Fundulus heteroclitus* ovum by fertilization is followed by formation of a block to polyspermy in the form of a "fertilization plug" consisting of numerous globules of various sizes. It would follow that if a type of "plug" were formed upon artificial activation, it would also prevent access of sperm to the ovum and would render such ova unfertilizable.

From SEM views of the micropyles it is clear that upon artificial activation the micropyle becomes blocked with globular, particulate, or fibrillar particles. There seems to be no definite correlation between the type of block and whether the egg has been artificially activated. We are unsure why 40% of the eggs which were uninseminated in 15‰ had micropylar blocks when experimental data indicate that they are almost all fertilizable. It is possible that the fixative could have activated some of the eggs.

Brummett and Dumont (1979, 1981a) have shown that cortical vesicle breakdown and extrusion of the contents begins to occur one second after insemination. They examined the contents of these vesicles, finding large (9 μ) and small (1.25 μ) granules, and still smaller globular and particulate matter. Dumont and Brummett (1980) also noted the pores around the micropyle but did not think that they perforated the chorion. Nevertheless, it may be possible that the explosive extrusion of granules from the cortical vesicles sends material out through the pores (which were found around all observed micropyles) and also thereby blocks the micropyles.

It is also possible, if the pores do not perforate the chorion, that the fibrils we observed were actually the inner lining of the pores turned inside out by the explosive activation process. It is likely that activation of PC ova by sea water involves the same cortical vesicle breakdown as activation by sperm.

There is evidence that the high salinity must interfere with sperm-egg interaction prior to cortical vesicle breakdown. The reasons for this are that the effect of 30‰ salinity has not progressed far enough to prevent fertilization if the inseminated eggs are transferred to 15‰ within one minute. Yet, 88% of the eggs failed to fertilize when stripped directly into a sperm suspension (in which sperm could certainly get to the eggs within the first minute). Thus, fertilization fails to occur in 30‰ salinity even when the sperm could have immediate access to the eggs. This strongly suggests that high salt somehow interferes with the direct sperm/egg interactions which lead to fertilization. Another possibility is that PC eggs might, upon contact with 30‰ water, secrete some substance which blocks sperm/egg interactions but which is dissolved by 15‰ salinity. No evidence for such a coating has been seen, however.

In considering the responses of the two populations to salinity, we should note the environments to which they have adapted. The salinities in which the parent fish live and have developed vary slightly between the two populations. However, Kinne (1962) suggests that from the evidence available, the induced differences due to spawning in varied salinity are not transmitted to the next generation. From these studies it would seem that killifish living in ~20‰ salinity in PC and fish living in ~25‰ salinity in SH should produce ova equally tolerant to varying salinities. Acclimation of the female fish to one or another salinity should not be responsible for the behavior of the ova.

Southampton is a relatively pristine area, while there is thermal, heavy metal, and oil pollution at Piles Creek, a tributary of the Arthur Kill. Gametes of fishes are expected to have only a short period of independent life, so the period of tolerance in nature would not have to exceed that required for successful fertilization. In nature, sperm and ova are codeposited and therefore the exposure to the medium, prior to fertilization, should be minimal. Given the conditions to which the PC population is exposed, *i.e.* presence of multiple pollution, it might be beneficial to the population if the ova were fertilizable for the shortest period of time possible, thus ensuring the lowest uptake of toxicants and possibly more successful embryonic development. The chorion becomes more impermeable over time (Tay and Garside, 1975) and thus more resistant to uptake of pollutants.

Weis *et al.* (1981) found that the PC embryos were more resistant to teratological effects of methylmercury than were embryos from Montauk or Southampton, which showed much greater variation in susceptibility, ranging from highly tolerant to highly susceptible. Perhaps the PC population has adapted narrowly to the specific conditions of its habitat and cannot tolerate changes in its environment. This might account for the inability of PC ova to fertilize in 30‰ (an "unnatural" environment). Furthermore, Renna (1982) noted that the PC adults had a much higher mortality in artificial sea water than the SH adults, which may also indicate a reduced ability to adjust. The PC fish seem to have adapted narrowly to the conditions of Piles Creek and to have lost some euryplasticity in the process.

Another possibility that should be considered is that the PC adults are under stress living in a polluted environment. Renna (1982) found that survival and fin regeneration rate of PC adults was much lower than SH adults in both clean water and methylmercury-contaminated water. This could indicate a weakened population of fish. Weakened adults might produce weakened gametes that might not be as adaptable as their counterparts from clean environments. The lack of adaptability

is true for ova but not for sperm in this case, since the ability of sperm to fertilize ova was not affected by salinity.

On the other hand, the differences between the ova response to salinity in the two populations might not be pollution-related at all, but may be a component of major differences in reproductive biology within this species. A variety of differences have been found which vary clinally along a north-south gradient. Southern populations of *F. heteroclitus* tend to produce eggs with more numerous, shorter chorionic fibrils (Brummett and Dumont, 1981b), more numerous oil droplets (Morin and Able, 1980), and to have their reproduction more closely tied to a lunar cycle than do more northern "races" (Wallace and Selman, 1981). Southern populations have been observed to deposit their eggs in *Modiolus* shells (Able and Castagna, 1975), a reproductive behavior not observed in more northern fish. Gene frequency differences have been noted (Place and Powers, 1978) which have been correlated with egg hatching times (DiMichele and Powers, 1982). The populations studied here are both from the intergrade zone, but in terms of oil droplet counts, chorionic fibrils, isozymes, and reproductive periodicity, the PC population tends to be closer to the southern type and the Long Island populations to the northern type (Heber, 1981). However, the fibers which we observed extending out of the pores around the micropyle of PC eggs were also observed by Brummett (personal communication) in eggs from Woods Hole, but not from South Carolina. Given the many differences in reproductive biology, it would therefore not be surprising if egg responses to salinity might also vary geographically.

Acknowledgments

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C.P.B. was an undergraduate honors student during the course of this research.

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